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Demonstration and partial purification of lactoperoxidase from human colostrum

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A peroxidase with stability, chromatographic and immunoreactive properties similar to that of bovine lactoperoxidase has been partly purified from human colostrum. Hydrophobic interaction chromatography on Phenyl-Sepharose Cl-4B gave a 10-fold purification with an apparent recovery of about 45%. The enzyme was quantitatively and specifically adsorbed to beads of anti-lactoperoxidase (bovine)-Protein A-Sepharose. No adsorption of the enzyme was observed on immunoadsorbent columns prepared with high-titre polyclonal antibodies raised against human myeloperoxidase and human eosinophile peroxidase.

Lactoperoxidase *Human colostrum* *Protein A-Sepharose* *Myeloperoxidase* *Eosinophile peroxidase*

1. INTRODUCTION

Human breast milk contains many enzymes, some of which may assist in digestion or have other functions [1]. Particular interest has been focused on the possible presence of a lactoperoxidase antimicrobial system in human milk as an important factor in the defence against infections (review [2–4]). However, no true secretory peroxidase has so far been isolated from human milk, and the question of whether human milk contains a secretory peroxidase as proposed [5], or if the activity can be accounted for by myeloperoxidase present in neutrophile granulocytes, is not yet answered. In a recent biochemical study it was concluded that there is no detectable lactoperoxidase in human milk, and that the peroxidase activity found in human colostrum is attributed to myeloperoxidase derived from leukocytes in the milk [6]. Here, we report on the demonstration and partial purification of a peroxidase from human colostrum, which has a stability as well as chromatographic and immunoreactive properties similar to that of lactoperoxidase isolated from bovine milk [7].

2. MATERIALS AND METHODS

2.1. Preparation of human colostrum and leukocytes

Samples of human colostrum, obtained from 4 healthy mothers 2–4 days after delivery, were pooled and kept frozen at −25°C until used. Fat was removed by centrifugation at 38 000 × *g* for 45 min at 4°C. Casein was partly removed by adjusting the pH to 4.6 [8], followed by precipitation for 1 h at 0°C and centrifugation at 38 000 × *g* for 45 min at 4°C. The pH of the resulting whey was adjusted to 7.0, and concentrated Na-acetate was added to match the buffer (2 M Na-acetate, pH 7.0) used to equilibrate the Phenyl-Sepharose column (see section 2.2),

Leukocytes were prepared from blood of healthy donors [9].

2.2. Chromatography on Phenyl-Sepharose

Hydrophobic interaction chromatography on a column (2 × 10 cm) of Phenyl-Sepharose Cl-4B (Pharmacia, Uppsala) was performed essentially as described for bovine lactoperoxidase [10], including equilibration of the column with 2 M Na-acetate, pH 7.0. The whey was centrifuged (12 000 × *g* for

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20 min at 4°C) and adsorbed at a flow rate of 0.05 ml/min, and the column washed with equilibration buffer. Isocratic elution was obtained with 0.05 M Na-acetate, pH 7.0, at a flow rate of 0.05 ml/min. The collected fractions (1.5 ml each) were analyzed for absorbance at 280 and 412 nm and peroxidase activity. The overall preparation time was 48 h.

2.3. Isolation of peroxidases

Bovine lactoperoxidase B was isolated essentially as described [7]. Human myeloperoxidase [11] and eosinophile peroxidase [11] were isolated as described, and generously supplied by Dr. Ragnar L. Olsen, Institute of Medical Biochemistry, University of Tromsø. The specific activities were 8.9, 15.2, and 50.8 U/mg protein for the purified lactoperoxidase, myeloperoxidase and eosinophile peroxidase, respectively.

2.4. Polyclonal antibodies

Polyclonal antibodies were raised in rabbits against bovine lactoperoxidase B by a standard immunization protocol. The immune-serum was used either without further purification or after purification by ammonium sulfate fractionation [12]; the partly purified IgG fraction was stored at −25°C at a concentration of 5.2 mg/ml (IgG) in 5 mM K-phosphate (pH 7.0) and 50 mM NaCl. 13.7 μg IgG completely removed 1 μg of bovine lactoperoxidase B. Polyclonal antibodies against human myeloperoxidase and human eosinophile peroxidase were generously supplied by Dr. Ragnar L. Olsen, Institute of Medical Biology, University of Tromsø. The anti-myeloperoxidase was purified by affinity chromatography on a myeloperoxidase-Sepharose column, and stored at a concentration of 0.31 mg/ml in 50 mM Tris (pH 7.4) and 0.15 M NaCl; 6 μg of the purified IgG completely removed the activity of 1 μg myeloperoxidase. The anti-eosinophile peroxidase was purified by fractional ammonium sulphate precipitation and DEAE-cellulose chromatography, and stored at a concentration of 1.0 mg/ml in 50 mM Tris (pH 7.4) and 0.15 M NaCl; 6 μg of the purified IgG completely removed the activity of 1 μg eosinophile peroxidase.

2.5. Protein A-Sepharose immunoaffinity columns

Protein A-Sepharose (Pharmacia) was swollen in 100 mM K-phosphate buffer, pH 8.0. For each adsorption experiment approx. 50 μl of swollen gel (equivalent to 15 mg dry wt) was preincubated with serum or purified IgG diluted in equilibration buffer, pH 8.0; 60–540 μg IgG in a total volume of 400 μl was used in each experiment. The suspension was gently stirred by a micropipette for 1 h at 0°C. After washing with the equilibration buffer (20 bed volumes ×5), the Sepharose beads were suspended in 120 μl of 100 mM K-phosphate buffer, pH 7.4, to which 60 μl of the antigen was added. The suspension was gently stirred by a micropipette for 1 h at 0°C (approx. 15 times), and the supernatant above the sedimented Sepharose beads was assayed for peroxidase activity. In experiments with highly purified peroxidases, the enzymes were diluted in media containing either unspecific milk proteins or bovine serum albumin.

2.6. Peroxidase activity

Peroxidase activity was measured at 470 nm by the guaiacol method [13] using a Cary model 219 spectrophotometer and the extinction coefficient ϵ ($mM^{-1} \cdot cm^{-1}$) = 26.6 for tetraguaiacol at 470 nm [14]. One unit of activity is defined as 1 μmol tetraguaiacol formed per min at 25°C.

2.7. Other analytical procedures

Protein was measured by the Coomassie brilliant blue method [15]. Bovine lactoperoxidase B was assayed spectrophotometrically using the extinction coefficient ϵ ($mM^{-1} \cdot cm^{-1}$) = 112.2 at 412 nm [16].

3. RESULTS

3.1. Peroxidase activity in human colostrum

In agreement with [5] we found that the peroxidase activity of freshly obtained colostrum varies greatly between donors, i.e., from almost zero to 12.6 mU/ml of skimmed milk in which the total peroxidase activity was removed by an immunoaffinity column of anti-lactoperoxidase (bovine) (see sections 2.5 and 3.3).

3.2. Chromatography on Phenyl-Sepharose CL-4B

When 90 ml of pooled whey of human colostrum (see section 2.2), containing 153 mU peroxidase, was subjected to hydrophobic affinity chromatography on Phenyl-Sepharose Cl-4B, the total per-

oxidase activity was adsorbed to the column. A peak of absorbance at 412 nm and peroxidase activity was eluted (fig.1) under conditions and with a retention time characteristic of bovine lactoperoxidase [10]. The elution profile at 412 nm revealed a major peak corresponding to the peak of peroxidase activity (21.0 mU/ml in the peak fraction), but additional components with absorbance at 412 nm were also observed with retention times shorter than the peroxidase; the absorption spectra indicated the possible presence of flavoproteins (not shown). Additional milk proteins eluted with the peroxidase activity as indicated by the $A_{280\,nm}$ absorbance profile (fig.1).

It should be mentioned that the peroxidase in human colostrum also binds to the lectin column ConA-Sepharose, and is eluted from this column in a manner similar to that observed for bovine lactoperoxidase (not shown). Furthermore, when a crude preparation of approx. 740 mU of myeloperoxidase, extracted from human leucocytes, was processed as described in section 2.2 and chromatographed on Phenyl-Sepharose, no activity was recovered in the eluted material.

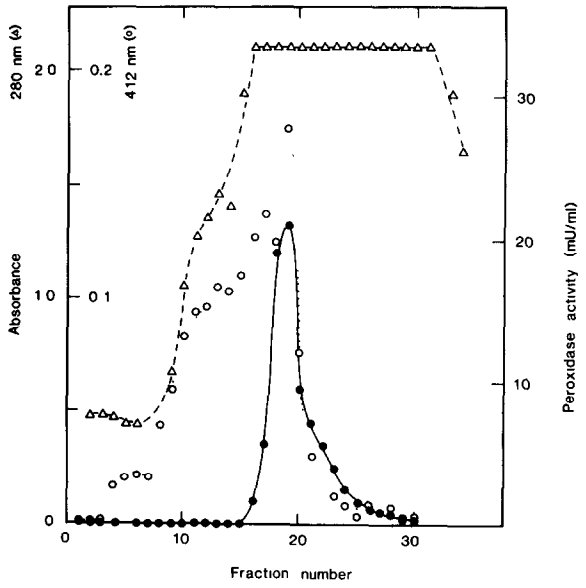

Fig.1. Elution profile of lactoperoxidase from human colostrum on Phenyl-Sepharose Cl-4B chromatography. The absorbance at 280 (△) and 412 (○) nm as well as peroxidase activity (●) were measured in each fraction.

3.3. *Immunological studies*

The fractions collected from the Phenyl-Sepharose Cl-4B column containing the highest peroxidase activity were used for the immunoadsorption experiments. As seen from fig.2, 0.3–2.4 mU of the isolated peroxidase (i.e., the peak fraction in fig.1) was found to be quantitatively adsorbed to the anti-lactoperoxidase (bovine)-Protein A-Sepharose beads at an antibody:antigen ratio (by weight) of 2000:1 or higher. No adsorption of the enzyme was obtained with the corresponding preimmune serum or with the polyclonal antibodies raised against human myeloperoxidase (the total binding capacity of the immunoadsorbent was estimated to be 10.0 μg or 152 mU of specific peroxidase) or human eosinophile peroxidase (the total binding capacity of the immunoadsorbent was estimated to be 16.7 μg or 848 mU of specific peroxidase).

In order to further test the specificity of our polyclonal antibodies against bovine lactoperoxidase, adsorption experiments with highly purified human myeloperoxidase and human eosinophile peroxidase were also performed; the purified enzymes were stabilized by added unspecific milk proteins (see section 2.5). None of the eosinophile

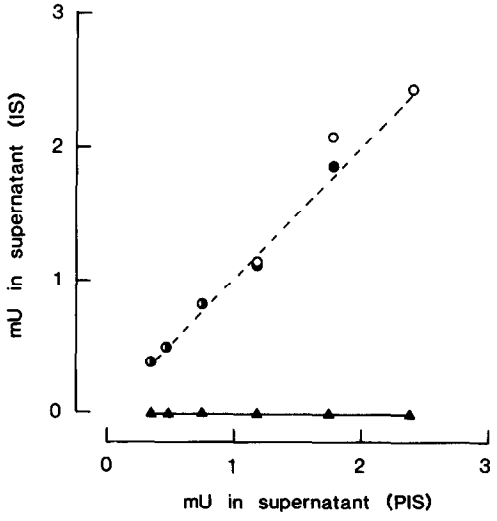

Fig.2. Immunoadsorption of partly purified lactoperoxidase from human colostrum to beads of Protein A-Sepharose (▲) prepared with anti-lactoperoxidase (bovine) immune serum (IS) or with preimmune serum (PIS). Controls with purified human myeloperoxidase (●) and purified human eosinophile peroxidase (○).

peroxidase (0.10 μg or 5.0 mU of enzyme and an antibody:antigen ratio (by wt) of 5400:1) was adsorbed to the beads of anti-lactoperoxidase (bovine)-protein A-Sepharose, whereas the myeloperoxidase (0.38 μg or 5.8 mU of enzyme) was adsorbed to about 20% (18–22%, $n = 6$) at an antibody:antigen ratio (by weight) of 1420:1.

4. DISCUSSION

Peroxidase activity was first demonstrated in human milk by authors in [5]. Based on stability studies of the enzyme it was concluded that the peroxidase was similar to bovine lactoperoxidase and therefore represented a genuine secretory peroxidase, and possibly also an important defence factor in human milk. This conclusion has more recently been challenged by biochemical studies [6] concluding that there is no detectable lactoperoxidase in human milk, and that the peroxidase activity often found in human colostrum is due to peroxidase derived from milk leukocytes.

Here, both chromatographic and immunologic experiments support the presence of a true secretory peroxidase (lactoperoxidase) in human colostrum. The chromatographic behaviour of the peroxidase on Phenyl-Sepharose Cl-4B, as determined by the absorbance at 412 nm and peroxidase activity (fig.1), was similar to that of bovine lactoperoxidase [10], and we have also reached a similar conclusion based on affinity chromatography on a lectin column of ConA-Sepharose (data not shown), indicating that the human milk peroxidase is a glycoprotein, as is the bovine enzyme [7]. It should also be mentioned that the enzyme is quite stable, including exposure to both low pH (4.6) and high ionic strength (2 M Na-acetate). These conditions result in a rapid decay of myeloperoxidase when added to skimmed milk preparations at concentrations about 5 times higher than corresponding to the endogeneous peroxidase activity in the colostrum (not shown). Most significant, however, was the finding that the isolated peroxidase from human milk reacts with anti-lactoperoxidase (bovine), but not with anti-myeloperoxidase (human) and anti-eosinophile peroxidase (human) (fig.2).

The recovery (45% based on activity measurements) of 67 mU peroxidase (equivalent to 7.5 μg bovine lactoperoxidase) from 90 ml of pooled colostrum explains why this peroxidase has remained undetected for so long, and particularly the negative conclusions of authors in [6] who based their immunological studies on immunodiffusion experiments only. Although our results have shown that human milk peroxidase activity is due to a true secretory peroxidase (lactoperoxidase), further studies on the purified enzyme will be required to demonstrate its chemical properties.

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